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Table of Contents

Cover.....	p 1
SF 298.....	p 2
Table of Contents.....	p 3
Introduction.....	p 4
Body.....	p 4-9
Key Research Accomplishments.....	p 9
Reportable Outcomes.....	p 9
Conclusions.....	p 10
References.....	p 10-11

SUBJECT: Annual Report for Award Number DAMD17-02-1-0159

“Hepatocyte Growth Factor and Interleukin-6 in Prostate Cancer Bone Metastasis”

INTRODUCTION: The hypothesis of this grant proposal is that androgen-ablative therapy paradoxically increases growth factor secretion from bone stromal cells and that this may stimulate the growth of prostate cancer metastases. We proposed to test this hypothesis using Interleukin-6 (IL-6) and hepatocyte growth factor/scatter factor (HGF/SF) as paradigms of androgen regulated growth stimulators. However, in two separate experimental systems – human bone stromal cells and mouse bone and bone marrow – we were unable to demonstrate that androgen-deficient conditions cause an increase in IL-6 or HGF/SF gene expression. As a result of this initial negative investigation in task 1 we decided to take a broad approach for identifying changes in gene expression upon androgen deprivation of mouse bone. Using a specific mouse DNA array platform enriched in sequences that are expressed in mouse prostate and bone, we discovered multiple genes that become up-regulated upon castration. We selected those genes that may regulate growth factor systems involved in the crosstalk between the bone environment and prostate cancer cells. One of these is the IL-7 receptor, which generates a possible link to increased IL-6 production in the androgen-deprived bone marrow environment.

BODY: Summary of work completed so far during the funding period of the award:

Task 1 (months 1-12) was “To determine whether hepatocyte growth factor/scatter factor synthesis by bone stromal cells is regulated by androgen”. To test this hypothesis we conducted (1) measurements of HGF/SF mRNA by RT-PCR in normal and orchiectomized mice and did not find an increase in HGF/SF upon androgen withdrawal. However, we detected increased Met (HGF/SF receptor) expression in orchiectomized mice by Western blotting.

For this task, we developed a. a method to extract intact RNA from mouse bone; b. a semi-quantitative RT-PCR to measure HGF, IL-6, the bone specific transcription factor: Cbfa-1, and osteocalcin; Using this methods, we were not able to detect significant differences in gene expression between normal and orchiectomized bone.

Thus we conclude, that IL-6 and HGF/SF are not significantly regulated in the bone and bone marrow of C57/BL6 mice by androgen.

In **task 2** (months 12-36), we planned “To determine whether IL-6 regulates HGF/SF synthesis in the murine bone stroma and in cultured human bone cells”. Since we were not able to demonstrate up-regulation of IL-6 or HGF/SF RNA in orchiectomized mice, we did not move to studying the effects of IL-6 in cultured mouse bone stromal cells. As the androgen receptor (AR) field has matured over the past three years, the gene expression changes caused by expression of AR protein in in-vitro cultured cells frequently do not recapitulate in-vivo gene activation by the AR. Further, it is not understood what the target genes of the AR might be in bone stromal cells. Thus we prefer not to proceed with experiments of expressing the AR in human bone stromal cells without available measurements to assess AR function.

Because we believe that we can obtain the most interesting and relevant data by examining the androgen regulation of the human bone marrow environment in a truly in-vivo system, we initiated a collaboration with the bone marrow transplantation team at FHCRC. The animal Core facility of the Center of Excellence in Hematology has established an animal model of a mouse reconstituted with human bone marrow. This system is ideally suited for our study of androgen regulation of HUMAN bone marrow and will spend the remaining time of the grant, exploring changes in IL-6 and other human cytokines in mice reconstituted with human bone marrow stromal cells and human hematopoietic bone marrow. We expect to include those data in the final report of the grant. In the past year, we have worked on the analysis of effects of androgen-ablation on mouse bone and bone marrow (task 4).

Considering the current state of the androgen receptor and bone metastasis fields, we are exploring androgen-regulated changes in IL-6 and HGF/SF gene and protein expression in an in-vivo system of human bone marrow and bone marrow stromal cells and will report on these findings in next year's report.

In **task 3** (months 24-36), we proposed "To determine the role of IL-6 as a mediator of the orchiectomy triggered HGF/SF synthesis". We have collected serum from mice treated with orchiectomy and other androgen-ablative treatments and will determine the effects of these treatments on serum IL-6 and HGF/SF levels.

We will analyze IL-6 and HGF/SF levels in sera from mice treated with various forms of androgen-ablative therapies.

Task 4: DNA array of mouse bone and bone marrow m-RNA, Months: 24-36

Goal of task 4: Identifying androgen regulated and IL-6-regulated growth factors in mouse bone.

The hypothesis of this grant proposal is that androgen-ablative therapy increases the synthesis of growth factors in mouse bone, either directly, or indirectly through IL-6. Further, we reasoned based on published reports that one of the androgen-suppressed growth factors could be HGF/SF. However, experiments in task one did not support androgen regulation of HGF/SF in C57BL6 mice. Therefore we decided

1. to survey the entire transcriptome of mouse bone for its androgen regulation using a prostate and bone-specific c-DNA array platform
2. to translate and confirm array results directly to human tissues.

The move to the Fred Hutchinson Cancer Research Center has opened novel translational avenues that did not exist at Cornell Medical College, when this grant was written. We decided to take advantage of novel technological advances that are available within the environment at FHCRC to address the important biological question of androgen-regulation of bone and bone marrow. We now realize that androgen-regulation and the IL-6 and HGF/SF growth factors may not only play a crucial role in the late stages of metastatic disease, when tumor cells are in contact with the bone, but also in early metastasis, when cancer cells are surrounded by bone marrow and bone marrow stroma. Thus we initiated two collaborations to obtain human tissue samples that can be used address changes in IL-6 and HGF/SF expression and to discover additional androgen-regulated genes in human bone marrow and bone. Through the prostate SPORE program, human tissues from prostate cancer metastases are collected by a rapid autopsy service team. These can be used to directly translate the results from the mouse models to human tissues. In addition, we initiated a collaboration with the bone marrow transplantation group. This group has a mouse model of NOD/SCID mice reconstituted with human bone marrow. In this unique animal model, human bone marrow can be subjected to hormonal manipulation in a highly controlled fashion.

Based on the hypothesis that orchiectomy triggers the synthesis of growth factors in the bone, we undertook a broad analysis of changes in the bone-associated transcription profile after androgen-deprivation using PEDB arrays.

Experimental methods used in Task 4:

Isolation of mouse bone and bone marrow: C57BL/6J mice were purchased from the Jackson Laboratory at 8 weeks of age. Mice were either castrated or sham-operated at week 7. Mice were kept for 10 weeks after

shipment at FHCRC before euthanasia and tissue collection. Femurs, tibiae and humeri are collected and cleaned from attached muscles and tendons. Bone marrow is separated from the bone by flushing several times with phosphate buffered saline and pelleted by centrifugation at 3000 rpm for 5 minutes. Bone marrows for RNA extraction are stored in TRIZOL™ at -80°C. Bones are snap frozen in liquid nitrogen and stored at -80°C.

Mouse prostate cDNA arrays (mPEDB) as a platform for measuring gene expression in bone. Arrays of DNA molecules offer a high-throughput, quantitative method for measuring differences in RNA expression levels between treated and untreated mice. Thousands of genes can be assessed simultaneously and subjected to a rigorous statistical analysis. The transcriptome of the mouse prostate has been expanded through sequencing of prostate-derived expression sequence tags (EST). 5000 prostate genes are represented within the (mouse Prostate Expression Data Base) mPEDB array, in addition to another 5000 random genes¹. We reasoned that using a mouse prostate array platform is a good starting point for analyzing androgen-regulated expression changes in bone, since (1) both tissues are regulated by androgen, (2) prostate cancer favors growth in bone, suggesting similarities between the bone and prostate microenvironments, and (3) up-to-date annotation of genes on the array is available through the bio-informatic team of the Nelson lab.

RNA isolation : Long bones from normal and orchietomized mice were separated into bone and bone marrow. RNA was extracted from bone marrow or pulverized bone using TRIZOL® (Invitrogen). The RNA is further cleaned and DNase-I treated using RNeasy® kit (Qiagen®). Quality of purified RNA was evaluated using OD_{260/280} ratio and an Agilent 2100 bioanalyzer (Agilent Technologies). Sixty to 100µg of RNA was extracted from bone marrow and 5 to 10µg from the bone of each mouse. Since RNA from bone was not sufficient for array studies, 2µg RNA were amplified in one round using RiboAmp® RNA Amplification Kit (Acturus), which generated approximately 40 µg bone-derived amplified RNA (aRNA). RNA and aRNA were stored in aliquots at -80 °C.

RNA labeling and array hybridization: Either 30 µg of total RNA or 2 µg of aRNA are reverse transcribed with oligo-dT primers or random hexamers with amino-allyl-dUTP added using SuperScript® II, RNase H minus Reverse Transcriptase. Amino-allyl-cDNA are labeled with Cy3 or Cy5 fluoro (Amersham) and hybridized to array at 63°C for 16 hours in SSC buffer with poly-A blockade. To exclude dye bias, a dye-swap is conducted for each paired sample. Fluorescent images are collected for both Cy3 and Cy5 by using a GenePix 4000A fluorescent scanner (Axon Instruments).

Analysis of array data: For each spot on the array, the Cy3 or Cy5 expression signal is defined as subtracting the median foreground with the median background. Flagged and poorly hybridized spots with intensity levels less than 2 standard deviations above the local background are excluded from analysis. Array data are normalized and analyzed using GeneSpring® 5.0 software (Silicon Genetics). Intensity dependent locally weighted regression scatter plot smoothing (Lowess) is applied to eliminate dye-related artifacts in two-color system. Measurements on each chip are also normalized to 50 percentile of the value on the chip to allow inter-array comparison. To evaluate significantly differentially expressed genes, a p-value of 0.05 in t-test is used as a cut-off.

Animal treatment: To determine whether the regulation of androgen-sensitive genes is reversible if we administer testosterone and how the genes are regulated by other androgen-ablative treatments, we treated animals with testosterone or diethyl-stilbesterol (DES). DES was administered as an implant of one 5 mg-60 day slow release pellet (83 µg/day) and bones were collected after 4 weeks. Testosterone was reconstituted in castrated mice by implantation of a 5mg – 21day slow release pellet (238 µg/day) and bone was collected after 2 weeks. Bone and bone marrow RNA transcripts were measured on the mPEDB array.

Results from experiments in task 4:**Results of c-DNA array analysis contrasting gene expression in bones of orchiectomized young and old mice, testosterone treated and DES treated mice.**

Raw DNA array data are illustrated on a scatter plot and only arrays that show sufficient quality and a narrow linear distribution of spots are used for further analysis. In high quality spotted DNA arrays, the individual spots of the scatter plot lie on a curve with minimal concavity. The outliers are highly reproducible amongst the mouse pairs within the same experiment. The scatter plots indicate that the differences in the transcriptomes of the bone and bone marrow in the castrated and sham-operated mice are quite small as most outlier genes are less than a two-fold difference in the castrated compared to sham-operated mice. In contrast, in the DES experiment, many genes were noted to be highly differentially regulated.

Orchiectomy treatment: Young mice: Data analysis revealed a total of 108 genes that are differentially expressed in androgen-deprived and normal bone. 98 were up-regulated in orchiectomized bone and 10 were down-regulated. Annotation was possible for 60/108 genes. 32 genes were more than 1.5-fold increased and 15 of these genes are shown in **Table 1**. The up-regulated genes were functionally classified into three groups: group-I is linked to tumor growth in bone (12 genes), group-II is connected to the B-cell hyperplasia that occurs with androgen-deprivation (10 genes) and group-III is unrelated (10 genes). Several of the overexpressed genes may be expressed in bone and bone marrow stromal cells and facilitate activation of specific growth-regulatory mechanisms involved in the interactions between bone and bone marrow and prostate cancer cells (**Table 2**). Some of the genes (IGFBP-5, Sox-4) are now to be expressed in mesenchymal cells of the bone and bone marrow. Old mice: Because prostate cancer occurs more commonly in older men and because there is a strong age-related distribution and component of aging in prostate cancer development, we analyzed the effects of androgen ablation on bone marrow in mice 1 year after castration/sham-operation. As shown in Table 1, there was good concordance between the genes up-regulated in young and old mice and in bone and bone marrow.

Testosterone treatment: in a preliminary DNA array analysis, 51 genes were down-regulated. Annotation was possible for 39/51 genes. 41 of 51 genes corresponded to genes for which opposite regulation was observed between castration and testosterone supplementation.

DES treatment: 1959 genes are differentially expressed including 830 up-regulated and 1129 down-regulated. Histologic analysis of DES treated sections of bone revealed massive new bone formation after DES treatment at the 83 µg/day concentration. Nevertheless, gene expression changes in 12 genes were observed in common between the DES, castration and androgen supplementation groups. In addition, there were 72 genes in common between the DES and the castration groups and only 3 genes in common between the DES and androgen supplementation group. Although there may be more overlapping genes, once more arrays of androgen supplemented animals are available, these data suggest that commonalities in gene expression changes exist between castration and DES treatments.

Flutamide treatment: We first conducted a dose titration of Flutamide. At a dose of 50µg/day, we observed approximately 50% reduction in the size of the seminal vesicles and anterior lobe of the prostate. However, there were no consistent gene expression changes in the four pairs of mice that we analyzed. Because castration and DES reduce the size of the seminal vesicals and prostate by about 90%, it is possible, that Flutamide only causes incomplete inhibition of the AR at the dose we used. We were unable to increase the dose because of toxicity to the animals. However, it is also possible, that the effects of castration are primarily due to a lack of estrogen. We are exploring this important possibility further.

Luprolide treatment: Our attempts to inhibit androgen production with Luprolide failed, even at a dose that corresponds to 100-times of the human dose. In a dose titration experiment, we observed an increase in prostate size, even at the highest drug concentration. We will therefore analyze the effects of a GnRH-receptor inhibitor in the near future.

Table 1. Differential expression of annotated and androgen-sensitive genes.
(genes with expression differences of > 1.5)

Systematic	Description	Castration		testosterone		DES
		Young Bone Marrow	Young Bone	Old Bone & Marrow		
Igfbp5	insulin-like growth factor binding protein 5		1.8		1.0	1.7
Vpreb3	pre-B lymphocyte gene 3				-6.7	-1.6
Pou2af1	POU domain class 2 associating factor 1		1.6		-4.4	-1.5
Pafah1b3	platelet-activating factor acetylhydrolase isoform 1b alpha1 subunit		1.7	1.9	-3.8	-1.4
Sox4	SRY-box containing gene 4		1.5	1.6	-3.6	
Cd79b	CD79B antigen	2.0	1.7		-4.0	-2.2
Bach2	BTB and CNC homology 2	2.0			-4.7	-1.9
Lef1	lymphoid enhancer binding factor 1	1.9	1.7	1.7	-2.6	NA
Sbk	SH3-binding kinase	1.9	1.7	1.7	-3.5	-1.2
Il7r	interleukin 7 receptor	1.9		1.8	-3.5	-1.4
Foxo1	forkhead box O1	1.8	1.5	1.7	-2.5	-1.2
Glicc1	glucocorticoid induced transcript 1	1.6	1.6	1.5	-2.6	-1.2
Blk	B lymphoid kinase	1.6	1.7	1.7	-2.9	NA
Scd1	stearoyl-Coenzyme A desaturase 1	1.6	1.9		-1.7	1.6
Siglec10	sialic acid binding Ig-like lectin 10	1.6	1.6	1.9	-2.7	-2.3

Functional properties of androgen-sensitive genes:

Table 1. Genes up-regulated by androgen-deprivation in mouse bone that potentially relate to the stimulation of growth and invasion of metastatic prostate cancer.

	Genes (abbreviation)	Functional category	Effects on metastatic tumor
1	IGFBP5 ²	increases IGF activity, unique receptor	increases tumor growth
2	PAF acetylhydrolase (Pafah1b3) ³	Decreases pro-apoptotic PAF	stimulates survival and invasion of tumor cells
3	SRYbox gene 4 (Sox4) ⁴	transcription factor, patterning	Mesenchymal growth factors
4	interleukin 7 receptor ⁵	IL7R triggers IL-6 synth. by BM stroma	Incr. tumor growth through IL-6
5	serine (or cysteine) protease Inhibitor ,cladeH (Serpinh1) ⁶	= hsp47, stablizes and increases collagen secretion from stromal cells	stimulates tumor growth through increasing cell adhesion
6	Thrombomodulin ⁷	receptor for activation of protein C	Incr. growth through active prot. C
7	Clusterin (Clu) ⁸	secreted protein stimulating cell survival	stimulates tumor cell survival
8	Amyotrophic lateral sclerosis 2 (ALS2) ⁹	GEFfor Rab and Rho, regulating vesicular transport and cytoskeleton	promotes secretion of GFs from bone
9	Platelet factor 4 (PF4) ¹⁰	binds FGF and stabilizes activity	Incr. tumor growth through FGF
10	Cxcl12, chemokine lig. ¹¹	binds to CXCR4 on tumor cells	tumor cell activation and invasion
11	cAMP-phosphodiesterase 4B (PDE4B) ¹²	Decr. cAMP, promotes cytokine secretion, PDE4B inhibitor effective in MM	promotes tumor growth
12	Osteonectin/SPARC ¹³	ECM protein stim. migration and osteoblast differentiation	tumor invasion and tumor-bone interactions

Abbreviations: IGFBP: insulin growth factor binding protein; PAF: platelet activating factor; Sox 4: SRY Box gene 4, GF: growth factor.

Other genes were significantly increased (>1.5 fold) and are related to the B-cell expansion, triggered by androgen-ablative therapy. These are: The pre-B lymphocyte gene 3 (VpreB3), a B-cell specific transcription factor¹⁴; Fc receptor related protein X (FcRX), a cell surface receptor for B-cell activation¹⁵; immunoglobulin-associated beta (Igb); BTB and CNC homology 2 (Bach2), a B-cell specific transcription factor¹⁶; heavy chain of IgM (Igh6); SH3 binding kinase (Sbk)¹⁷, forkhead box 1 (Foxo1) and B-lymphoid kinase (Blk)^{18,19}; lymphoid-restricted membrane protein (Lrmp)²⁰; and the transcription factor E2F2a²¹. Several significantly overexpressed genes were of uncertain relationship to tumor growth. These include myosin light chain alkali cardiac atria²², stearoyl-Coenzyme A desaturase 1 (Scd1)²³, erythrocyte protein band 4.1-like 4b (Epb4.114b)²⁴, C-type lectin-like receptor 2²⁵ and f-box and leucine-rich repeat protein 12 (Fbxl12)²⁶.

Interpretation of data:

Our data show that castration causes the up-regulation of genes in the mouse bone. Several of the genes can be linked to the promotion of tumor growth in the bone environment. Most notably, IGFBP-5, a positive regulator of insulin-like growth factor (IGF), and Sox-4, a mesenchymal transcription factor that may regulate the fibroblast growth factor axes, are up-regulated after androgen ablation. Recent data show, that various androgen-ablative treatments may differently effect the bone and bone marrow environment. Therefore, by understanding which growth promoting gene expression changes are treatment specific, it may be possible to prevent these by hormonal manipulation. In addition, it is possible that gene expression changes during the therapeutic response differ amongst patients and that it may be possible to tailor treatments to individuals. In summary, we obtained data that support the main hypothesis of the grant proposal. **Our data show that androgen-ablative therapy causes the expression of genes that can potentially promote the growth of prostate cancer metastases in the bone and bone marrow environment.**

KEY RESEARCH ACCOMPLISHMENTS:

- We identified the changes in the transcriptome of mouse bone resulting from androgen-ablation using a mouse prostate specific expression array.
- We demonstrated that about 75% of gene expression changes can be reversed by reconstituting androgen levels.
- We show good concurrence of gene expression changes in the bone and bone marrow of young and old mice after castration.
- We identified 98 genes that are up-regulated upon castration and 10 that are downregulated. We also have preliminary gene lists after treatment with DES and Flutamide,
- We found that the upregulated genes belonged to three broad categories: category 1 is associated with growth factor receptor systems and could contribute to androgen-independent growth of prostate cancer cells in bone (12 genes); category 2 contains genes associated with the B-cell hyperplasia that is induced by castration (32 genes) and category 3 are all other genes and all genes that are not annotated.
- We identified IGFBP5 and Sox-4 as two genes that are up-regulated by androgen and that are associated with the IGF and FGF axis, respectively, making them excellent candidates for further studies of androgen-dependence in the bone/bone marrow stroma and potential stimulators of prostate cancer growth.
- We identified differences in gene expression changes in the bone and bone marrow after different androgen-ablative treatments

REPORTABLE OUTCOMES:

- The data generated in the mouse system were used as preliminary results in a post-doctoral grant application to the prostate cancer DOD program in 2004.
- 2004 AACR abstract # 172
- Prostate Cancer Foundation Abstract # 45

CONCLUSIONS:

Several conclusions related to the statement of work can be drawn from the data obtained in the last year:

1. androgen ablation induced gene expression changes that may regulate several different growth factor systems that may enhance metastatic growth.
2. IL-6 and HGF/SF were not amongst the genes that are significantly up-regulated by androgen ablation.
3. the reason for the lack of up-regulation of IL-6 and HGF/SF could be the mouse strain and we had proposed in the original grant to analyze two separate mouse strains.
4. since resources of human bone metastases have become available, it appears that analyzing expression human tissues would provide more information than analysis of another mouse strain.
5. we will generate a mouse model in which we can manipulate reconstituted human bone marrow to analyze hormonally induced gene expression changes in a highly controllable fashion.
6. the results obtained so far provided data for grant applications and will hopefully lead to a publication in the next year of funding.

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